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MICROCALORIMETRIC STUDIES ON THE INTERACTIONS OF LANTHANIDE IONS WITH BOVINE SERUM ALBUMIN

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The interactions of lanthanide ions (Ln^{3+}) with bovine serum albumin (BSA) under mimetic physiological conditions (310.15 K, pH 6.7, 0.1 M NaCl) were studied by microcalorimetry. For the first time, based on Two Sets of Independent Sites Model, molar enthalpies ($\Delta_r H_{m1}$, $\Delta_r H_{m2}$) and coordination number (n_1, n_2) of the two sets of binding sites with different affinity were obtained directly from the microcalorimetric results. It was shown that the interactions are endothermic and entropy-driving processes. By combining with fluorescence spectroscopy, other thermodynamic parameters ($\Delta_r G_{m1}$, $\Delta_r S_{m1}$) were determined for high-affinity specific sites.

Keywords: *bovine serum albumin (BSA), fluorescence spectroscopy, lanthanide ions (Ln^{3+}), microcalorimetry*

Introduction

Serum albumins are the most abundant proteins in blood plasma, accounting for about 60% of the total protein [1, 2] and provide about 80% of the osmotic pressure of blood [1]. They play an important role in the transport and deposition of a variety of endogenous and exogenous substances in blood [3] due to the existence of a limited number of binding regions with very different specificity [4]. In particular, its role in the transport of essential, toxic, and therapeutic metals has received considerable attention [5]. Bovine serum albumin (BSA), a protein with a molecular mass of 66 300, has been used in our study for its primary structure is very well known and its tertiary structure was determined [1, 6].

With the extensive applications of rare earths in agriculture, medicine and research in molecular biology etc., the lanthanide elements are introduced to biological bodies by various ways. The biological effect and mechanism of lanthanide elements have been widely paid attention to. In particular, great interest has been aroused in doing research on the lanthanide-protein complexes, because lanthanide ions possess some special functions in biochemistry [7–12]. For example, the interactions of lanthanide ions with serum albumins have been widely studied by various methods [13–18], including ultrafiltration, equilibrium dialysis, and spectroscopic measurements, etc. The binding characteristics achieved were that there are two sets of binding sites: one are specific ones, with high affinity and low binding capacity; and the other are non-specific ones, with low affinity and high binding capacity. The number of high-affinity binding sites and the binding constants of different binding sites have been reported [15]. But thermodynamic parameters were seldom reported. However, these thermodynamic parameters were indispensable because they can potentially provide valuable insight as to the nature of the interactions and the forces that stabilize the complexes.

Compared with classical methods, thermochemical method can provide in-situ, online, quasi-continuous, non-invasive and accurate measurements of the thermodynamic parameters of the reaction under investigation. Moreover, there is also no constraint on both the solvent and the spectral, electrochemical, or other properties of the reaction systems involved. Owing to these advantages, the thermochemical method has received increasing attention from researchers in many fields. Since the absorption or production of heat is an intrinsic property of all reactions, calorimetry as the most powerful tool of the thermochemical method has been extensively used [19–22]. In particular, calorimetry has been widely employed to the researches on the binding of metal ions to proteins [23–26].

In this work, we systemically studied the binding reactions of lanthanide ions (Ln^{3+}) to bovine serum albumin (BSA) under mimetic physiological conditions (310.15 K, pH 6.7, 0.1 M NaCl) on LKB-2107 batch microcalorimeter. According to the microcalorimetric results, number of binding sites (n_1, n_2) and apparent reaction enthalpy ($\Delta_r H_{m1}$, $\Delta_r H_{m2}$) for different-affinity

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binding sites of Ln^{3+} on BSA were obtained directly. Furthermore, combining with fluorescence spectroscopic studies, other thermodynamic parameters ($\Delta_r G_{m1}$, $\Delta_r S_{m1}$) were determined for high-affinity specific binding sites.

Experimental

Materials

BSA (fractionV, from Roche Chemical Co.) was used without further purification. The concentration of BSA was determined by the method as previously described [19].

Lanthanide chlorides were prepared from their oxides (99.9%, from China Medicine Group, Shanghai Chemical Reagent Corporation), and their pH values were adjusted to about 6. The concentrations of lanthanide ions (III) were titrated with EDTA.

Other chemicals used were of analytical grade. Doubly distilled water was used throughout. All measurements were performed in 0.1 M NaCl, pH 6.7 and 310.15 K in order to simulate physiological conditions.

Methods

LKB-2107 batch microcalorimeter system combines a micro-batch reactor (BR) with a conduction calorimeter. All fluorescence measurements were carried out on a Perkin-Elmer LS 55 Luminescence Spectrometer equipped with a Xenon discharge Lamp and 1.0 cm quartz cells and a thermostat bath. All pH measurements were made with a digital pH-meter (Orion 828) with a combined glass-calomel electrode.

Microcalorimetric measurements

The calorimetric curves were obtained at 310.15 K by an LKB-2107 batch microcalorimeter system. Before each calorimetric experiment, all reagent solutions were diluted to the required concentrations with 0.1 M NaCl solution (pH 6.7).

In experiments, 2.00 mL lanthanide chlorides solution was injected into reaction cell, and 4.00 mL BSA solution ($1.0 \cdot 10^{-4}$ M) was injected into reaction cell. In order to avoid the influence of the heat effect of diluting and mixing etc., 2.00 mL 0.1 M NaCl solution was injected into reference cell, and the same addition was made to reference cell as reaction cell. And in order to obtain the dilution heat effects of rare earth chlorides (Q_d), 2.00 mL lanthanide chlorides solution was injected into reaction cell, and 0.1 M NaCl solution was injected into other three cells. When the microcalorimeter system had been in thermal equilibrium and a steady baseline was obtained on the recorder, the reaction run was initiated by rotating the calorimeter 360° clockwise and counterclockwise, respectively, so as to fully mix the solutions. The heat generated in the reaction process was detected in the form of thermal potential by thermoelectric piles, and the amplified output signal was recorded as the calorimetric curve by LKB-2210 dual-pen integrating recorder.

In microcalorimetric experiments, all determinations were made in duplicate. Moreover, a series of lanthanide chlorides concentrations were used.

Fluorescence measurements

Measurements of steady-state fluorescence were made on a Perkin-Elmer LS55 Luminescence Spectrometer equipped with a circulating water bath at 310.15 K. The fluorescence spectra were measured at a fixed protein concentration of $1.0 \cdot 10^{-5}$ M. Excitation and emission slits were set at 3 and 5 nm, respectively. The spectra were recorded in 280–550 nm range and the excitation wavelength was set at 288 nm.

Interactions of Ln^{3+} with BSA were studied using a fluorescence titration method. The protein solution with fixed concentration was titrated with an increment of microliter Ln^{3+} solution (5–50 μL each and total volume not exceed 200 μL).

Theory

Model for two sets of independent sites

The calorimetric data can be analyzed by a mass-action approach. The underlying assumptions are as follows: *i*) one protein molecule has two sets of sites, which can bind the same ligand. All sites within one set are thermodynamically identical, and the two sets of sites can be characterized by binding constant (K_1, K_2), standard molar binding enthalpy (ΔH_1, ΔH_2) and number of binding sites (n_1, n_2); *ii*) The two sets are assumed to be completely independent; *iii*) One is specific high-affinity binding sites which are preferentially occupied by ligands, and the other is nonspecific low-affinity binding sites which can bind ligands only after the specific ones have been fully occupied.

Based upon the above assumptions, there are two sets of Ln^{3+} binding sites on BSA that one is specific ones (K_1, ΔH_1, n_1) and the other is nonspecific ones (K_2, ΔH_2, n_2).

Methods for determination of coordination number and thermodynamic parameters

For 1: *n* binding mode of protein (P) with small ligand (L), the apparent binding constant *K* for a single site can be expressed as:

$$K=\frac{[PL_n]}{(P_t-[PL_n])(n[PL_n])} \quad (1)$$

where P_t, L_t refer to total concentration of protein and small ligand, respectively; $[PL_n]$ refers to the concentration of protein-ligand complex.

Given fixed protein concentration, there exists an equation

$$Q_t=\Delta_r H_m L_b V \quad (2)$$

where Q_i, $\Delta_r H_m$ refer to the P–L binding reaction heat and the apparent molar binding enthalpy of a single site, respectively; L_b concentration of the bound ligand and V the volume of reaction system (in this paper, V=6.00 cm^3).

From Eq. (1), L_b can be expressed as:

$$L_b=P_t nKL_f/(1+KL_f) \quad (3)$$

where L_f refers to concentration of the unbound ligand and K the apparent binding constant.

The relation between L_b, L_t and L_f is

$$L_t=L_b+L_f \quad (4)$$

Combining Eqs (2), (3) and (4), the relation between Q_i and L_t can be deduced

$$Q_i=[A-(A^2-4nP_tL_t)^{1/2}]\Delta_r H_m V/2 \quad (5)$$

where

$$A=1/K+nP_t+L_t \quad (6)$$

Then, if K is more than 10^6, that is $1/K$ approximate zero, Eq. (5) can be turned into

$$Q_i=(nP_t+L_t-|nP_t-L_t|)\Delta_r H_m V/2 \quad (7)$$

when protein is unsaturated complexation ($nP_t>L_t$), Eq. (7) can grow into

$$Q_i=L_t\Delta_r H_m V$$

That is the apparent molar binding enthalpy $\Delta_r H_m$ can be obtained

$$\Delta_r H_m=Q_i/(L_t V) \quad (8)$$

when protein is saturated complexation ($nP_t<L_t$), Eq. (7) can grow into

$$Q_i=nP_t\Delta_r H_m V \quad (9)$$

Then combining with acquired $\Delta_r H_m$, coordination number n can be determined by

$$n=Q_i/(P_t\Delta_r H_m V) \quad (10)$$

Moreover, from the binding constants (K) and the standard molar binding enthalpy (ΔH), the standard molar binding free energy (ΔG) and the standard molar binding entropy (ΔS) can be calculated by the following relations, respectively:

$$\Delta G=-RT\ln K$$
$$\Delta S=\frac{\Delta H-\Delta G}{T} \quad (11)$$

Results and discussion

From all of the microcalorimetric curves obtained in this work, it was shown that the interactions of BSA with lanthanide ions are at rapid equilibrium:

$$BSA+nLn\leftrightarrow BSA\cdot Ln_n \quad \text{(rapid equilibrium)}$$

where Ln is lanthanide ions, and $BSA\cdot Ln_n$ the complex between BSA and Ln.

Figure 1 shows an example microcalorimetric curve for the interaction of BSA with lanthanide ions (La^{3+}). The calorimetric curve shows a quick endothermic peak, indicating that the apparent interaction enthalpy was positive under given conditions. It can be inferred that the interactions between BSA and Ln^{3+} are largely endothermic and entropy-driven process according to the enthalpy-entropy compensation law.

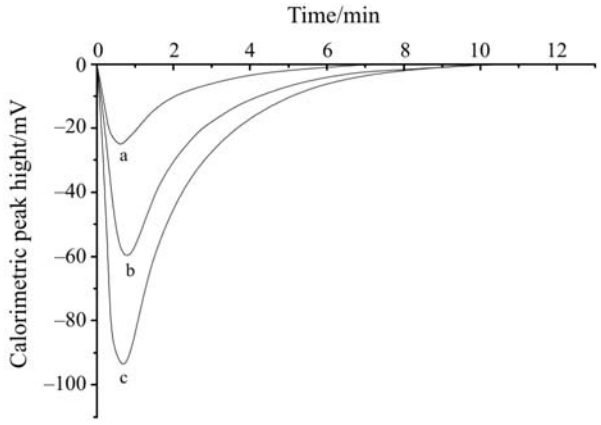

Fig. 1 Microcalorimetric curves for the binding reaction of BSA with La^{3+}; a – $C_{La}^{3+}=7.76\cdot10^{-5}$ mol L^{-1}; b – $C_{La}^{3+}=3.10\cdot10^{-4}$ mol L^{-1}; c – $C_{La}^{3+}=6.21\cdot10^{-4}$ mol L^{-1}. T=310.15 K, pH=6.7, [NaCl]=0.1 mol L^{-1}, $C_{BSA}=6.06\cdot10^{-5}$ mol L^{-1}

Determination of apparent molar reaction enthalpy $\Delta_r H_m$ and coordination number n

Under given conditions, the reaction heat effects of Ln^{3+} with BSA were obtained by fixing the protein concentration and varying the concentration of Ln^{3+}.

According to microcalorimetric results, Fig. 2 was obtained by plotting the pure reaction heat effect (Q_i) *vs*. the Ln^{3+}/BSA molar ratio (R). Obviously, all of curves occur in a biphasic manner with a characteristic inflection between the two sets of binding sites on BSA for Ln^{3+}. Qualitative similar binding curves obtained for all other Ln^{3+} are also biphasic.

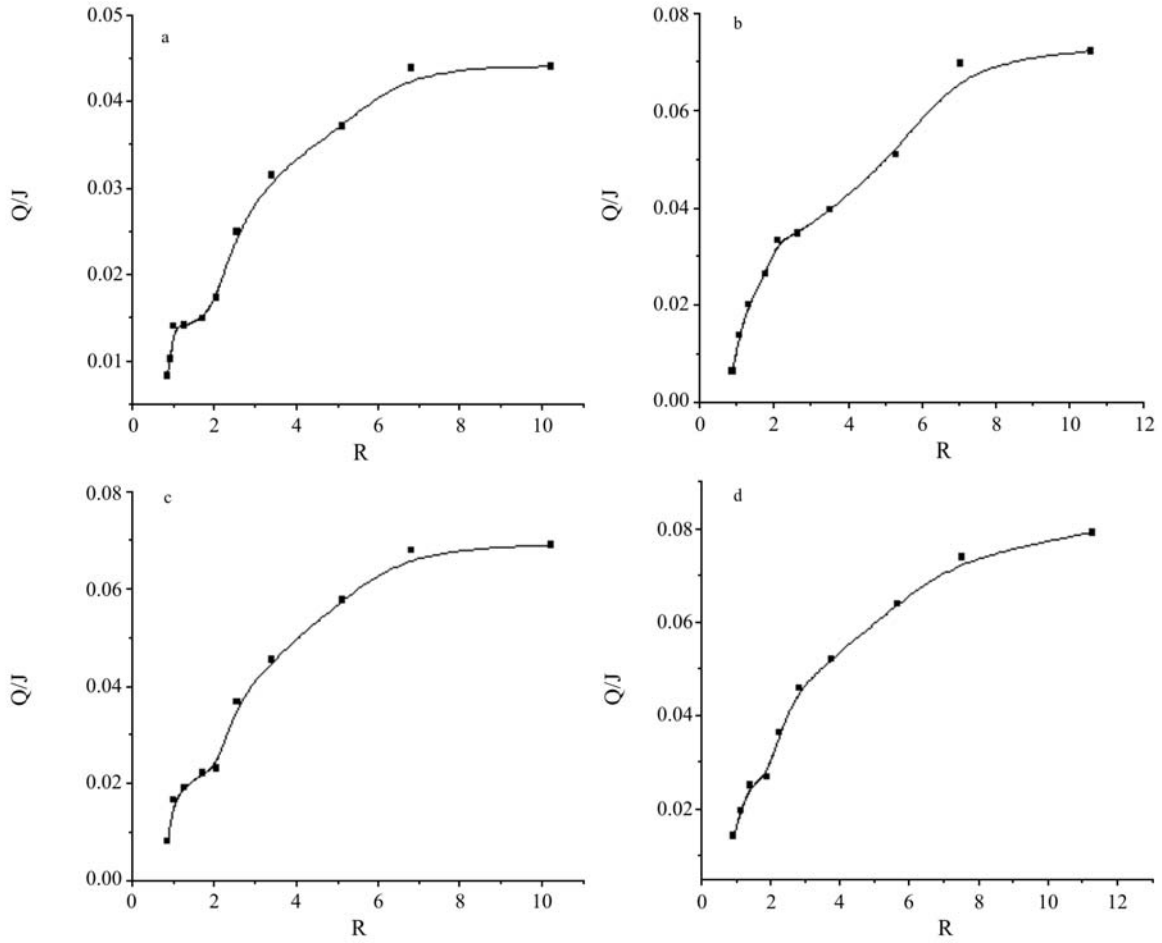

Fig. 2 The graphs of BSA-Ln^{3+} pure binding heat effects (Q_i) changing with Ln^{3+}/BSA molar ratio (R) increase; a – BSA-La^{3+}; b – BSA-Er^{3+}; c– BSA-Gd^{3+}; d – BSA-Yb^{3+}

Based upon the Two Sets of Independent Sites Model, it can be concluded that at low Ln^{3+} concentration, Ln^{3+} binds to the specific high-affinity sites (sites I) on BSA with Q_i increasing; and at certain concentrations (about one to two Ln^{3+} ions per protein molecule) these specific binding sites are fully saturated and Q_i approximately arrive at a fixed value (platform II); then at even higher concentrations, Ln^{3+} binds to the nonspecific low-affinity sites (sites II) with Q_i increasing; finally, these low affinity sites appear saturated when the total Ln^{3+} concentration exceeds a certain value and Q_i arrive at a new fixed value (platform II).

Then, apparent molar reaction enthalpy $\Delta_r H_{m1}$ of sites I can be determined by calculating several calorimetric data before platform I by Eq. (8)

$$\Delta_r H_{m1} = Q_i / (C_{Ln} V)$$

Subsequently, coordination number n_1 of sites can be obtained by Eq. (10)

$$n_1 = Q_I / (C_{BSA} \Delta_r H_{m1} V)$$

where Q_I refers to the heat effect of platform I.

Similarly, apparent molar reaction enthalpy $\Delta_r H_{m2}$ and coordination number n_2 of sites II can be obtained by calorimetric data before platform I and the heat effect of platform II (Q_{II}).

Table 1 presents the apparent molar reaction enthalpy ($\Delta_r H_{m1}$, $\Delta_r H_{m2}$) and coordination number (n_1, n_2) for La^{3+} binding to BSA.

The apparent molar reaction enthalpy ($\Delta_r H_{m1}$, $\Delta_r H_{m2}$) and coordination number (n_1, n_2) for different Ln^{3+} binding to BSA were obtained directly under mimetic physiological conditions (310.15 K, 0.1 mol L^{-1} NaCl, pH 6.7). Results are listed in Table 2.

Other thermodynamic parameters for the binding of lanthanide ions (Ln^{3+}) to BSA

Steady-state fluorescence spectroscopic studies was carried out by exciting the protein at 288 nm, monitoring the BSA intrinsic fluorescence intensity at 345 nm as a function of added Ln^{3+} with a view to elucidating the binding stoichiometry. Figure 3 showed that by plotting F_0/F (F_0 and F refer to the fluorescence intensity of BSA at 345 nm without Ln^{3+} and

Table 1 Results of the apparent molar reaction enthalpy and coordination number for La^{3+} binding to BSA*

$C_{La}/010^{-5}$ mol L^{-1}	R	Q_i/J	$\Delta_r H_{m,i}$/kJ mol^{-1}	$\Delta_r H_m$/kJ mol^{-1}	n_i	n
5.64	0.93	0.01024	30.48	$\Delta_r H_{m1}$=30.37		
6.21	1.02	0.01399	30.25			
7.76	1.28	0.01408		Platform I	1.28	n_1=1.3
10.34	1.71	0.01485			1.34	
12.41	2.05	0.01731	23.27			
15.51	2.56	0.02485	26.72	$\Delta_r H_{m2}$=23.81		
20.69	3.41	0.03146	25.33			
31.03	5.12	0.03707	19.91			
41.37	6.83	0.04382		Platform II	5.06	n_2=5.1
62.06	10.24	0.04400			5.08	

*Annotation: R refers to Ln^{3+}/BSA molar ratio; Q_i the pure heat effect of reaction (Q_i=$Q_i^{'}$–Q_d, where $Q_i^{'}$ refers to the total heat effect of reaction and Q_d the corresponding dilution heat effect of rare earth chlorides); $\Delta_r H_{m,i}$ is the calculated apparent molar reaction enthalpy corresponding to a given R; The apparent molar reaction enthalpy of sitesland sites II ($\Delta_r H_{m1}$ and $\Delta_r H_{m2}$) equal to the average value of $\Delta_r H_{m,i}$ before Platform l and II, respectively; n_i the calculated number corresponding to a given R of Platform; The coordination number of sitesland sitesII (n_1 and n_2) equal to the average value of n_i corresponding to Platformland II, respectively

Table 2 Apparent molar reaction enthalpy ($\Delta_r H_{m1}$, $\Delta_r H_{m2}$) and coordination number (n_1, n_2) for 11 kinds of Ln^{3+} binding to BSA

Ln^{3+}	Z_{RE}	Specific high-affinity sites (sites I)		Nonspecific low-affinity site (sites II)	
		n_1	$\Delta_r H_{m1}$/kJ mol^{-1}	n_2	$\Delta_r H_{m2}$/kJ mol^{-1}
La^{3+}	57	1.3	30.37	5.1	23.81
Pr^{3+}	59	1.8	42.56	4.9	33.95
Nd^{3+}	60	3.0	31.98	6.8	23.74
Sm^{3+}	62	3.2	35.65	7.6	24.65
Eu^{3+}	63	1.7	33.38	6.6	23.16
Gd^{3+}	64	1.5	42.94	5.6	33.82
Tb^{3+}	65	2.2	36.10	5.7	31.65
Dy^{3+}	66	1.8	33.61	6.6	28.50
Er^{3+}	68	2.4	43.47	6.8	31.51
Tm^{3+}	69	2.1	43.20	6.5	29.98
Yb^{3+}	70	1.6	45.90	6.1	34.52

with Ln^{3+}, respectively) against the Ln^{3+}/BSA molar ratio, the fluorescence intensity at 345 nm increased linearly with Ln^{3+} addition until the Ln^{3+}/BSA molar ratio reached a certain value where the titration curves break off. Qualitative similar titration curves were obtained for all other Ln^{3+}. Collectively, it demonstrates that there are at least two sets of Ln^{3+}-binding sites on BSA as previously described [27]. The coordination number of specific binding sites (n_1) corresponds to the Ln^{3+}/BSA molar ratio of break off points (shown in Table 3). It was shown that the values of n_1 correspond to those obtained by microcalorimetry.

Based upon the two sets of independent sites model proposed in this paper, the specific binding sites were saturated and the $BSA{\cdot}Ln_{n1}$ complex can be formed at the break off point:

$$BSA + n_1 Ln^{3+} \rightarrow BSA{\cdot}Ln_{n1}$$

It can be obtained that

$$F/F_0 = ([BSA]_{total} - [BSA{\cdot}Ln_{n1}])/[BSA]_{total}$$

$$[Ln^{3+}] = [Ln^{3+}]_{total} - n_1[BSA{\cdot}Ln_{n1}]$$

$$[BSA] = [BSA]_{total} - [BSA{\cdot}Ln_{n1}]$$

Then, the apparent binding constants K_1 for specific binding sites can be expressed as

$$K_1 = [BSA\,Ln_{n1}]/([Ln^{3+}]^{n1}[BSA]) = (1-F/F_0)/\left\{([Ln^{3+}]_{total} - n_1[BSA]_{total} + n_1 FF_0^{-1}[BSA]_{total})^{n1} FF_0^{-1}\right\} \quad (12)$$

By Eq. (12), the apparent binding constants K_1 of specific sites (sites I) under mimetic physiological conditions (310.15 K, pH 6.7, 0.1 M NaCl) were obtained (Table 3). Then, other thermodynamic parame-

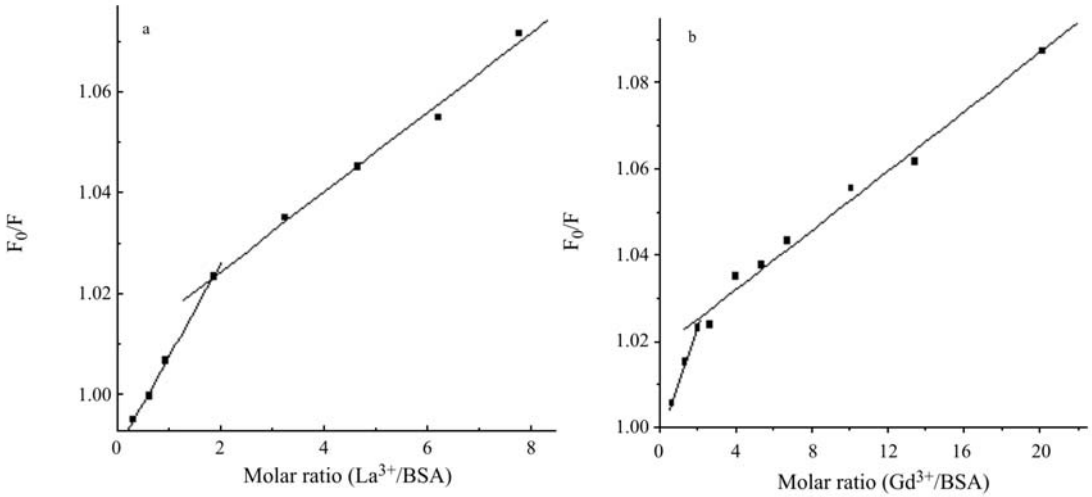

Fig. 3 Fluorescence titration curves (λ_{ex}=288 nm): the fluorescence intensity of BSA at 345 nm; a – with the La^{3+}/BSA molar ratio and b – with the Gd^{3+}/BSA molar ratio

Table 3 Coordination number n_1, apparent binding constants lgK_1 and other thermodynamic parameters of specific sites for 11 kinds of Ln^{3+*}

Ln^{3+}	n_1	lgK_1	$\Delta_r G_{m1}$/kJ mol^{-1}	$\Delta_r S_{m1}$/J K^{-1} mol^{-1}
La^{3+}	1.9	7.90	–46.91	249.17
Pr^{3+}	2.2	8.98	–53.32	309.14
Nd^{3+}	3.5	11.80	–70.06	329.00
Sm^{3+}	3.0	12.11	–71.90	346.77
Eu^{3+}	2.1	8.46	–50.23	269.58
Gd^{3+}	2.1	6.80	–40.37	268.61
Tb^{3+}	2.0	8.81	–52.31	285.06
Dy^{3+}	2.2	14.36	–85.26	383.27
Er^{3+}	2.1	8.87	–52.66	309.95
Tm^{3+}	1.9	9.74	–57.83	325.75
Yb^{3+}	2.0	10.69	–63.47	352.69

*Annotation: $\Delta_r G_{m1}$ and $\Delta_r S_{m1}$ refer to the standard molar binding free energy and the standard molar binding entropy for the specific high-affinity sites (sites I)

ters ($\Delta_r G_{m1}$ and $\Delta_r S_{m1}$) for specific sites were calculated by Eq. (11) (shown in Table 3).

Results showed that the apparent molar reaction enthalpy $\Delta_r H_m$ are all positive ones. Then it was further demonstrated that the binding process of Ln^{3+} to BSA is endothermic, and is accompanied by entropy increasing (as shown in Table 3).

As Evans [28] pointed out that in aqueous solution Ln^{3+} generally exist as complex ions. For H_2O tend to coordinate with Ln^{3+}, the ubiquitous complex is $[Ln(H_2O)n]^{3+}$. With other ligands coexisting, they will probably compete with H_2O for Ln^{3+}. While only under appropriate concentration, the oxo-ligands or chelates can complex with Ln^{3+}, and the complex formed often contain water molecules. And it was further indicated that the properties of complex ions are important for lanthanide ions in aqueous solution.

A reasonable analysis for $\Delta_r H_m$ all positive can be reached with the assumption that the total heat effect of coordination reaction between Ln^{3+} and BSA divides into two parts, namely the hydration-bond-breaking heat effect and the coordination-bond-forming heat effect. The former dehydration process is endothermic, while the latter coordination process is exothermic. When the positive effect of the former process on the enthalpy change exceeds the negative effect of the latter one, the overall enthalpy change ΔH is positive. It is confirmed that the coordination reaction is an entropy-driven process according to the enthalpy-entropy compensation law.

And as Zhang [15] described that Ln^{3+} exist in the form of hydrated ions in aqueous solutions, and that the outer-sphere of BSA molecules combine with one hydrated layer. Besides, the ligand-forming radi-

cals of protein frequently form salt bridges ($-COO^{-}...^{+}H_3N-$) through electrostatic force. When Ln^{3+} coordinate with the ligand-forming radicals of BSA, there are solvation effect and electrostatic effect that must be overcome, and as a result there exhibit endothermic and entropy increasing.

Conclusions

In this work, the interactions of lanthanide ions (Ln^{3+}) with bovine serum albumin (BSA) under mimetic physiological conditions (310.15 K, pH 6.7, 0.1 M NaCl) were systemically studied on LKB-2107 batch microcalorimeter. According to the microcalorimetric results, number of binding sites (n_1, n_2) and apparent reaction enthalpy ($\Delta_r H_{m1}$, $\Delta_r H_{m2}$) for different-affinity binding sites of Ln^{3+} on BSA were obtained directly for the first time. It was shown that the interactions are endothermic and entropy-driving processes. Furthermore, combining with fluorescence spectroscopic studies, other thermodynamic parameters ($\Delta_r G_{m1}$, $\Delta_r S_{m1}$) were determined for specific binding sites.

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